Bacterial Enzymes for Lignin Depolymerisation: New Biocatalysts for Generation of Renewable Chemicals from Biomass

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Abstract

The conversion of polymeric lignin from plant biomass into renewable chemicals is an important unsolved problem in the biorefinery concept. This article summarises recent developments in the discovery of bacterial enzymes for lignin degradation, our current understanding of their molecular mechanism of action, and their use to convert lignin or lignocellulose into aromatic chemicals. The review also discusses recent developments in screening of metagenomic libraries for new biocatalysts, and the use of protein engineering to enhance lignin degradation activity.

Introduction

Lignin is an aromatic heteropolymer comprising 15-30% of the lignocellulose cell wall of plant biomass, and is the most abundant source of renewable aromatic carbon in the biosphere. Given the need to reduce greenhouse gas emissions in 21st century society, there is considerable academic and commercial interest in finding new sustainable biocatalytic routes to fuels and chemicals from renewable sources of carbon such as plant biomass [1]. For aromatic chemicals, lignin is an obvious starting point, but is a very challenging polymer to deconstruct, due to the presence of non-hydrolysable ether C-O and C-C bond linkages, poor solubility in aqueous solution, and other technical challenges [2].

The search for microbial enzymes to deconstruct lignin has until recently focussed on white-rot basidomycete fungi such as *Phanerochaete chrysosporium*, that produce extracellular lignin peroxidases, manganese peroxidases, and multi-copper laccases that can attack lignin (see Figure 1) [3]. However, these fungal enzymes are often challenging to express in high yield, and their fungal hosts are not readily amenable to genetic modification for metabolic engineering, hence since 2010 there has been a resurgence of interest in lignin-oxidising enzymes from soil bacteria. A number of soil bacteria have been identified that can depolymerise lignin, mainly in the actinobacteria and α- and γ-proteobacteria phyla, albeit less rapidly than the most active basidiomycete fungi [4,5]. This article will describe recent developments in the enzymology of bacterial lignin-degrading enzymes, our current understanding of how they attack lignin, and applications for biotransformation.

1. Identification & characterisation of bacterial enzymes for lignin depolymerisation

Figure 1. Classes of fungal and bacterial lignin-degrading enzymes, showing structures and properties.

The first bacterial lignin-oxidising enzyme to be identified was peroxidase DypB from *Rhodococcus jostii* RHA1, a member of the dye-decolorising peroxidases, found in bacteria and fungi [6]. Dyp-type peroxidases have activity for dye decolorisation, but also oxidation of a range of phenolic substrates [7]. There are four sub-classes A-

D, based upon sequence alignment, of which classes A-C are found in bacteria, and class D found in fungi [7]. Although many Dyp-type peroxidases have been identified, only some have been demonstrated to have activity for oxidation of polymeric lignin. Lignin-oxidising Dyp peroxidase enzymes have been identified in *Amycolatopsis* sp. 75iv2 (DypC) [8], *Pseudomonas fluorescens* Pf-5 [9], and *Thermobifida fusca* [10]. Pfl Dyp1B is able to release an oxidised lignin dimer from wheat straw lignocellulose in the presence of Mn^{2+} [9]. The ability to oxidise polymeric lignin *in vitro* correlates in most cases with an ability to oxidise Mn^{2+} by some B-type and C-type Dyp peroxidases [6,8,9], although some A-type Dyps can oxidise lignin model compounds [10].

One of the best-studied hosts for bacterial lignin degradation is *Pseudomonas putida*, a well characterised aromatic degrader that has been verified via different experimental approaches to break down lignin [4,11], and has been used as a host for metabolic engineering of lignin bioconversion [12]. Lin et al have recently published a genomic and proteomic analysis of *P. putida* A514, implicating the role of two Dyp-type peroxidase enzymes in lignin breakdown [13]. They report that neither of these Dyp enzymes have activity for Mn^{2+} oxidation [13]. A Dyp-type peroxidase has also been reported from *Pseudomonas* sp. Q18 that is able to degrade alkali lignin [14].

In the actinobacteria, Dyp-type peroxidase from *Saccharomonospora viridis* DSM 43017 has been reported that is active for dye decolorisation at neutral-alkaline pH, unlike most such peroxidases whose optimum pH is typically 3-4, and has been applied to bleaching of eucalyptus kraft pulp [15]. A 38 kDa DyP-type peroxidase has also been reported from *Rhodococcus* sp that has been applied to kraft pulp bleaching [16]. Detailed mechanistic studies have been reported for a B-type Dyp peroxidase from *Enterobacter lignolyticus* [16]. A kinetic isotope effect of 2.44 was observed using D_2O_2 indicating that cleavage of the O-H bond during the formation of the compound I iron-oxo intermediate is rate-limiting, and an inverse solvent kinetic isotope effect was interpreted as evidence for a kinetically significant conformational change [17].

The second class of bacterial lignin-degrading enzymes are multi-copper oxidase enzymes, or laccases, which are best characterised from fungal sources, but have also been found in bacteria. Bacterial multi-copper oxidase from *Streptomyces coelicolor* A3 has been implicated in lignin breakdown, from gene deletion studies in which decreases in acid-precipitable lignin (APPL) were observed [18]. Overexpression of a

related SLAC enzyme in *Amycolatopsis* sp 75iv3 has been shown to lead to 6-fold increases in APPL production, with enhanced syringyl (S) content in the APPL structure, and the release of monocyclic aromatic products such as vanillin, 4-hydroxybenzoic acid, and 1,4-dihydroxy-3,5-dimethoxybenzene (via aryl-Cα cleavage) [19]. A blue multi-copper oxidase CueO from a lignin-degrading *Ochrobactrum* sp. strain has been characterised kinetically, and its crystal structure determined, showing slight differences in the type I copper centre, compared with fungal laccases [20]. The *Ochrobactrum* CueO was found to catalyse oxidative dimerization of lignin model compounds, but depolymerised lignosulfonate, generating vanillic acid as a product [20]. Multi-copper oxidase CopA enzymes from *Pseudomonas putida* and *P. fluorescens* Pf-5 have been found to be pseudo-laccases, requiring addition of exogenous Cu^{2+} for activity, and show stoichiometry of 8-10 mol Cu/mol for the holoenzyme, but show similar catalytic properties to other bacterial multi-copper oxidases [21]. Multi-copper oxidases CueO and CopA are also linked to bacterial copper utilisation, so it is likely that they have multiple cellular roles [20,21]. Furthermore, multi-copper oxidase complex Mnx has been demonstrated to oxidise Mn(II) to Mn(IV), and the catalytic mechanism for this process elucidated [22]. A multi-copper oxidase enzyme from *Paenibacillus glucanolyticus* SLM1 has also been kinetically characterised against phenolic substrates [23].

A novel lignin-oxidising manganese superoxide dismutase enzyme has been identified in lignin-degrading *Sphingobacterium* sp. T2 [24], which is able to solubilise organosolv and kraft lignin, and generate a mixture of polymeric and monocyclic aromatic products [24]. Recent studies on this enzyme have shown that the enzyme is able to generate hydroxyl radical via one-electron reduction of hydrogen peroxide, a reaction not normally observed in superoxide dismutase enzymes. The predominant reaction with polymeric lignin is demethylation of the methoxy aryl-substituents, and two amino acid replacements close to the Mn(II) centre were shown to be essential for this reactivity, causing increased solvent access to the Mn(II) centre [25].

The fourth group of lignin-degrading bacterial enzymes is the family of glutathione-dependent β-etherase enzymes, which catalyse the reductive cleavage of the β-aryl ether linkage via attack of glutathione at the β position of an oxidised aryl

unit containing an α ketone group. These enzymes were first identified in *Sphingobium* SYK-6, a bacterium with the ability to degrade a range of lignin dimers that are likely to be lignin oxidation products, where dehydrogenases LigD and LigL catalyse oxidation of the benzylic α-hydroxyl group, LigE, LigF are stereospecific β-etherase enzymes for ether cleavage, and LigG catalyses reductive elimination of glutathione to generate a benzylic ketone product [26-28]. Crystal structures of *Sphingobium* SYK-6 LigD, LigO, LigL, LigG have been solved, providing insight into mechanisms of catalysis by these enzymes [29]. Analogous dehydrogenases LigO and LigN and β-etherase LigP have been identified in *Novosphingobium*, and have been shown to catalyse stereospecific β-ether cleavage [30]. A Nu-class glutathione S-transferase was identified in *Novosphingobium* as a β-etherase enzyme [30], which has been shown to act as a glutathione lyase, which can cleaves both enantiomers of the intermediate glutathione adduct [31]. A β-etherase enzyme has also recently been identified in the white-rot fungus *Dichomitus squalens* [32].

Screening microbes isolated from a range of environments could yield novel enzymes for the degradation of lignin, however it is accepted that most environmental microbes are unculturable, and the methods used to isolate and characterise those that can be grown are time consuming and work intensive. Hence there is current interest in the use of biosensors for high-throughput screening of metagenomic DNA libraries, as shown in Figure 2. A biosensor comprises of a genetic regulatory unit, which is activated by a specific compound, coupled to a reporter gene, which gives a measurable output such as fluorescence or luminescence. Several biosensors have been developed for a range of compounds associated with the breakdown of lignin, including: vanillin [33,34], protocatechuate [35] and phenylpropenoic acids such as ferulic and p- coumaric acid [36]. These regulatory units have been discovered by screening regulator-reporter gene fusion libraries [33] or using known genetic regulation towards a target compound [36]. Rounds of mutagenesis and selection can be used to tighten the regulation towards a specific compound or improve the range of expression in the ‘on’ and ‘off’ states [35].

Genomic DNA is typically screened in ~40 kb fosmids in *E. coli*, incubated with lignin or lignin-like substrates, then the reporter strain containing the biosensor is added, and the output measurement taken to assess degradation of the substrate. Positive clones are then investigated to discover the genes responsible for activity.

The success rate of these strategies demonstrates their worth compared to traditional methods of screening. Using the vanillin and syringaldehyde sensor strain, Ho *et al*. screened 42,520 clones and had 147 positive clones [33]. Metagenomic analysis from lignin-treated sugarcane soil has also recently identified unculturable microbial sequences with lignin-degrading activity [37].

Figure 2. Biosensors for lignin-degrading enzyme discovery

2. Biotransformation of lignin by lignin-depolymerising enzymes

Our understanding of exactly how lignin-degrading enzymes attack polymeric lignin is still very incomplete. As shown in Figure 3, for the β-aryl ether structure which is the major structural unit found in polymeric lignin, there are a number of possible sites for oxidation or oxidative cleavage. In the relatively few cases where the site of reaction has been studied, studies may be based on the use of lignin dimer model compounds, or the site of reaction implied by structure of low molecular weight product released. A further complication is that products obtained are dependent on the type of lignin used: a study of the biocatalytic and chemocatalytic conversion of a set of lignins revealed different products from different lignin preparations, but showed that lignins containing high β-O-4 content released higher yield and number of products [38], providing some evidence that this linkage is probably the major site of attack in polymeric lignin.

For the bacterial Dyp-type peroxidases, the detection of vanillin from a lignin dimer substrate using *R. jostii* DypB indicated that Cα-Cβ oxidative cleavage (route B, Figure 3) had occurred, although dimerization via coupling of phenoxy radicals also occurred [6]. *P. fluorescens* Dyp1B had been shown to release products from Cα-Cβ cleavage (route B) or aryl-Cα cleavage (route C, Figure 3) from polymeric lignin substrates [38], and can release a lignin dimer product containing an oxidised ketodiol sidechain from treatment of wheat straw lignocellulose [9]. For *Sphingobacterium* MnSOD, the major site of reaction appears to be demethylation (route E, Figure 3) [25], but monomers arising from aryl-Cα cleavage and Cα-Cβ cleavage are also formed [24]. In model studies, oxidative cleavage by Dyp-type peroxidases has only been observed using units containing a free phenolic 4-hydroxyl group [6], therefore it seems likely that for breakdown of polymeric lignin

breakdown, they cleave from the ends of a lignin chain (exo-cleavage), rather than in the middle of a chain (endo-cleavage). Expression of *R. jostii* DypB in tobacco plants has been shown to yield 200% more fermentable sugars, and reduced lignin content, demonstrating that Dyp-type peroxidases can be expressed heterologously to depolymerise lignin [39].

The reaction of multi-copper oxidases (laccases) with lignin often results in repolymerisation via phenoxy radical formation (route D, Figure 3) [18-21]. However, in the presence of mediators such as 1-hydroxybenzotriazole (HBT) or methyl syringate, detailed NMR studies have shown that oxidation of the α-hydroxyl group of the β-aryl ether unit (route A, Figure 3) is a major reaction [40,41], however, studies of product release from lignin by fungal laccases has shown differences in behaviour depending on the mediator used [42]. Recent studies on *Amycolatopsis* SLAC have shown the release of products arising from aryl-Cα cleavage and Cα-Cβ cleavage [19].

Beta-etherase enzymes which catalyse reductive ether cleavage (route F, Figure 3) had until recently only been shown to act on lignin dimer substrates [26-31] that may be lignin degradation intermediates. Combination of LigE and LigF with glutathione lyase LigG was found to be effective for bioconversion of lignin models [43]. However, there have been two significant recent reports of β-etherase enzymes acting on polymeric lignin substrates. Picart *et al* have reported that treatment of beech wood lignin with laccase lcc2 M3 from *Trametes versicolor* in the presence of violuric acid, followed by β-etherases LigEG from *Sphingobium* SYK-6 and LigF from *Novosphingobium aromaticivorans*, yields a bio-oil containing low molecular weight aromatic compounds [44]. Furthermore, Gall *et al* have shown that a combination of β-etherases LigDEFN and $NaGST_{NU}$, together with glutathione reductase to recycle reduced glutathione, releases guiaiacyl (G), syringyl (S) and tricin units from lignin oligomers and polymeric lignin [45]. These reports raise the possibility that this class of enzymes could be used to attack polymeric lignin.

Figure 3. Different enzyme-catalysed reactions of β-O-4 unit found in lignin, showing different types of product, and enzymes reported to carry out that reaction.

3. Lignin degradation accessory enzymes

One limitation of using recombinant lignin-oxidising enzymes for *in vitro* biotransformation of lignin substrates is that dimerization or repolymerisation is often observed, due to the formation of phenoxy radicals that spontaneously recombine. Therefore it is likely that there are accessory enzymes *in vivo* that can trap phenoxy radicals via one-electron reduction. One candidate enzyme for this activity has been recently identified, a highly expressed extracellular dihydrolipoamide dehydrogenase from *Thermobifida fusca*, that has been shown to prevent dimerization of a lignin model compound *in vitro*, and change the profile of low molecular weight products formed [46]. Reductase enzymes such as this could be valuable accessory enzymes for lignin biotransformation.

Another accessory enzyme activity needed for lignin degradation *in vivo* is the generation of hydrogen peroxide co-substrate for lignin-oxidising peroxidase enzymes, probably generated from dioxygen by oxidase enzymes. Two such oxidase enzymes have been identified recently that appear to be linked to lignin degradation. A copper-dependent oxidase enzyme has been identified in *Thermobifida fusca*, that has been shown to result in reduced lignin content in sugarcane bagasse, and generates dilignol products [47]. A new pathway for metabolism of aryl-C_2 lignin fragments in *Rhodococcus jostii* RHA1 has also been shown to involve an FMN-dependent oxidase enzyme that can oxidise aldehyde intermediates and simultaneously generate hydrogen peroxide [48].

4. Protein engineering studies

Two recent reports describe the application of directed evolution methods to bacterial Dyp-type peroxidase enzymes. Brissos *et al* report the engineering of *Pseudomonas putida* DyP using error-prone polymerase chain reaction, giving a mutant enzyme containing three mutations (E188K, A142V, H12V), each on the surface of the enzyme (see Figure 4), that enhance k_{cat}/K_M for 2,6-dimethoxyphenol by 100-fold, and shift the optimum pH to 8.5 [49]. Rahmanpour *et al* report the use of focused libraries around the active site of *Pseudomonas fluorescens* Dyp1B, enhancing the k_{cat}/K_M for 2,4-dichlorophenol by 7-8 fold, and mutation H169L was found to enhance product release from polymeric lignin [50].

Figure 4. Amino acid residues identified in engineered DyP structures

In summary, the study of microbial lignin degradation is leading to the discovery of a range of new enzyme biocatalysts that could be applied either *in vitro* or in whole cell biotransformations for conversion of lignin to high value chemicals.

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Figure Legends

Figure 1. Active sites of lignin-degrading enzymes with important residues for catalysis. A. Fungal lignin-degrading enzymes: Lignin Peroxidase [1LLP] from *Phanerochaete chrysosporium* (W171, H47, R43, H176, E40, A36 and N182); Manganese Peroxidase [4CZO] from *Ceriporiopsis subvermispora* (H46, R42,H173, E40, E36, D175); Versatile Peroxidase [2BOQ] from *Pleurotus eryngii* (W164, H47, R43, H169, E40, E36, D175); laccase [3T6V] from *Steccherinum ochraceum* (H67, H402, H400, H452, C453, H454, H458, H397). B. Bacterial lignin-degrading enzymes; Dyp peroxidase [4HOV] from *Rhodococcus jostii* RHA1 (D153, R294, H226, E156, E239 and E215); Manganese Superoxide dismutase (SpMnSOD1) from *Sphingobacterium* sp. T2 [6GSB] (H26, H76, D163 and H167); β-Etherase LigF [4XT0] from *Sphingobium* sp. strain SYK-6 Glutathione (GSH) binding site (GSH, W148, Q144, H40, Q59, Q39, S13, E65 and S66); Multi-Copper Oxidase [4GXF] from *Streptomyces coelicolor* (H102, H104, H156, H231, H293, C288, H289, H287, H236 and H234). Graphics drawn using PyMol software.

Figure 2. Biosensors for lignin-degrading enzyme discovery. A. A biosensor comprises of a genetic regulatory unit, which represses the reporter gene promoter, and this repression is de-repressed by binding of the inducer molecule. Different gene regulators may have different specificity, and their specificity can be altered via protein engineering. B. This in turn leads to expression of the reporter gene, which gives a measurable output such as fluorescence or luminescence. C. Strains containing a library of environmental DNA are then incubated with lignin or lignin like substrates, and then the reporter strain is added and the reporter output is measured.

Figure 3. Different enzyme-catalysed reactions of β-O-4 guaiacyl (G) unit found in softwood lignin, showing different types of product, and enzymes reported to carry out that reaction.

Figure 4. Structures of engineered DyP-type peroxidases and location of residues which enhance their activity. A. Residues (L169 and L193) identified in *Pseudomonas fluorescens* Dyp1B using focused libraries. B. Residues (Y125, V142

and K188) found in *Pseudomonas putida* DyP using error-prone polymerase chain reaction. Models generated using Swiss-model (https://swissmodel.expasy.org/)

Figure 1

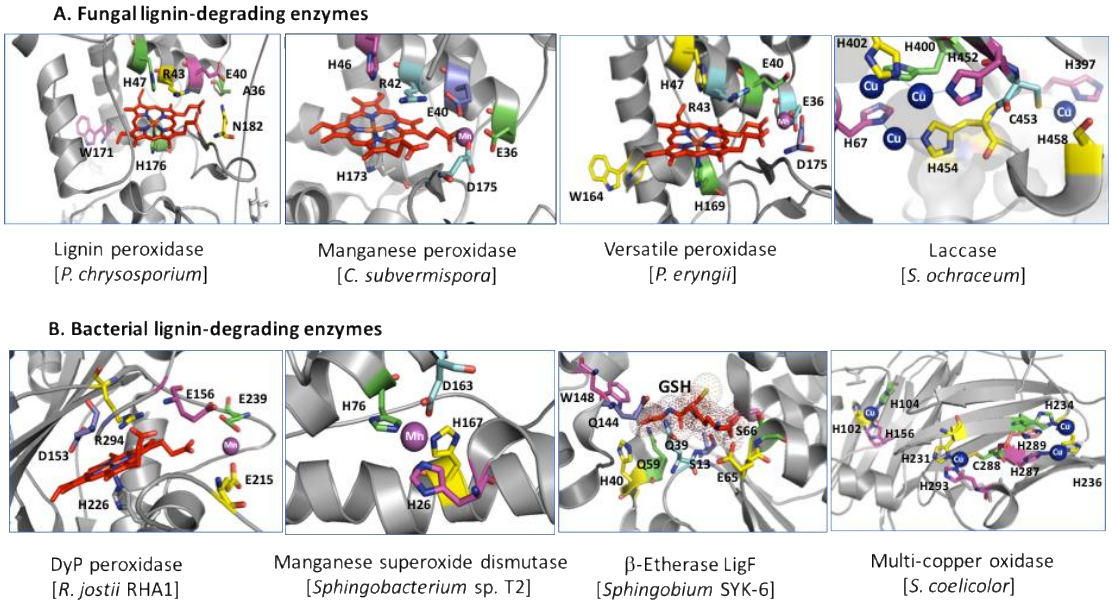


Figure 2

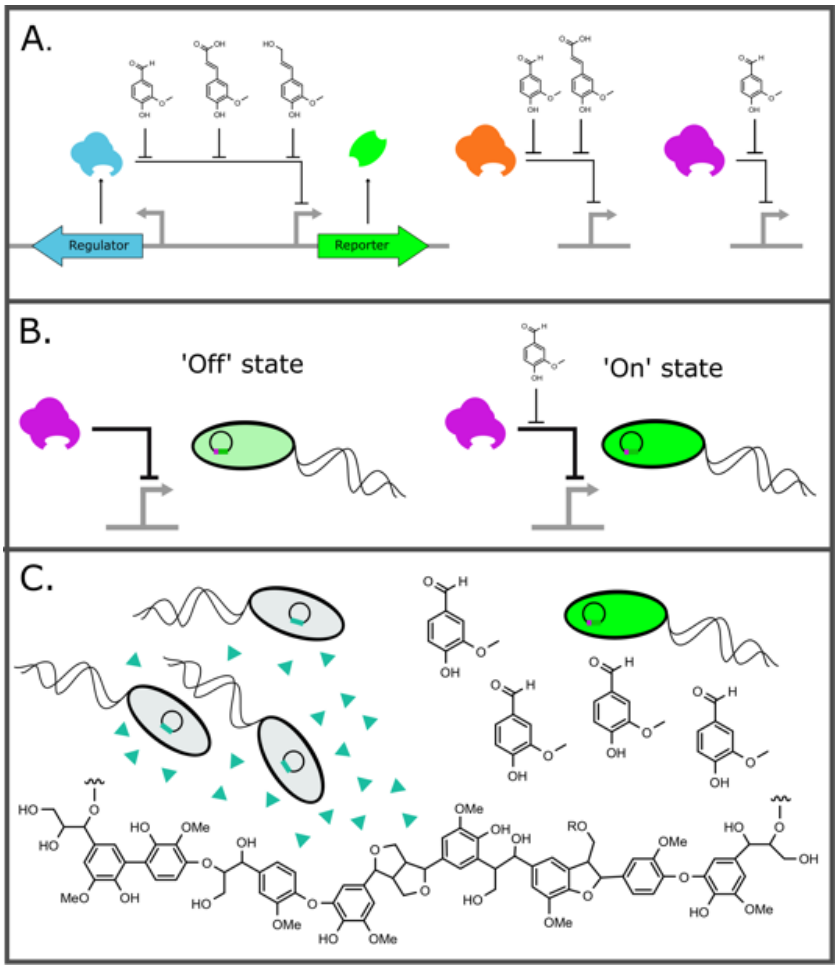
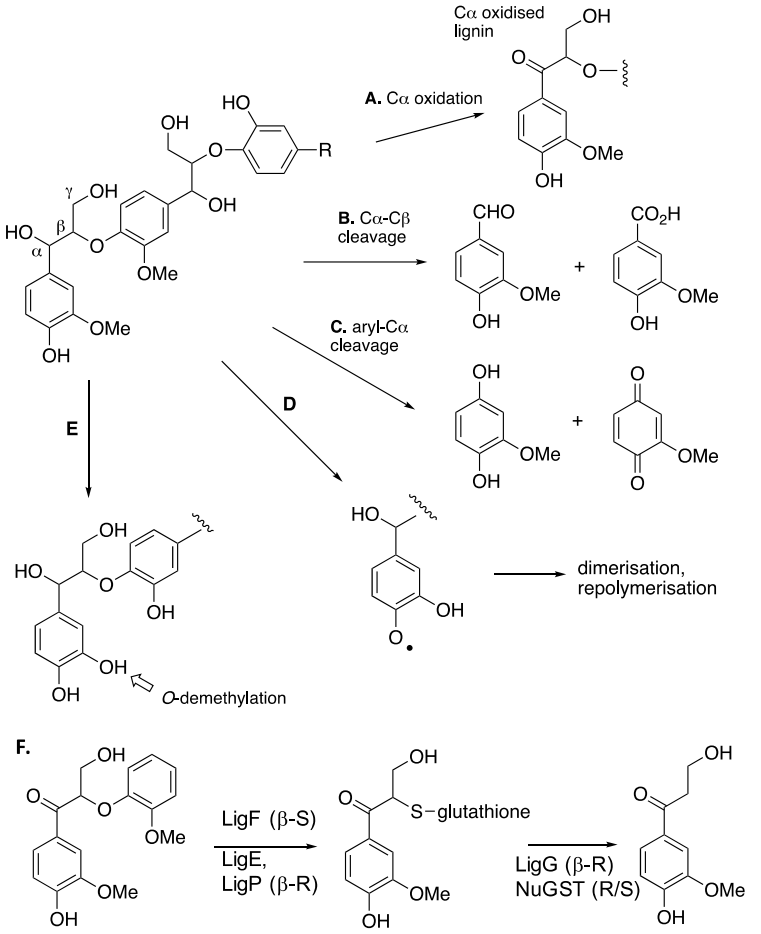


Figure 3



Reported for

Fungal laccases [40,41]

Fungal lignin peroxidase
R. jostii DypB [6]
P. fluorescens Dyp1B [38]

P. fluorescens Dyp1B [38]
Sphingobacterium MnSOD [24]
Amycolatopsis SLAC [19]

Fungal laccases
Ochrobactrum CueO [20]
P. putida CopA [21]

Sphingobacterium MnSOD [25]

Sphingobium SYK-6 LigEF [26,28]
Novosphingobium LigP [30,31]

Figure 4